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(57) Abstract

The present invention relates to reporter fusion proteins which allow the monitoring of facilitated transport and passive diffusion of proteins across the nuclear envelope in mammalian cells, cloning and expression vectors encoding these reporter proteins and transfected cell lines thereof. More particularly, the present invention relates to genes encoding reporter proteins to monitor transport and passive diffusion of proteins across the nuclear envelope in mammalian cells, which comprises a protein carrying a nuclear localization sequence (NLS) fused to at least one copy of a reporter protein, wherein the reporter protein allows for the visualization of the reporter fusion protein.

REPORTER FUSION PROTEINS, EXPRESSION VECTORS AND TRANSFECTED CELL LINES THEREOF FOR THE ANALYSIS OF NUCLEAR TRANSPORT

5   ## BACKGROUND OF THE INVENTION
       ### (a) Field of the Invention
              The invention relates to reporter fusion pro-
       teins which allow the monitoring of facilitated trans-
       port and passive diffusion of proteins across the
10   nuclear envelope in mammalian cells, cloning and
       expression vectors encoding these reporter proteins and
       transfected cell lines thereof.
       ### (b) Description of Prior Art
              The correct localization of proteins in
15   eukaryotes involves complex targeting reactions. These
       processes ensure that each cellular compartment con-
       tains an unique set of macromolecules required for the
       specialized function of the organelle. Proteins that
       are destined for the nucleus carry specific targeting
20   sequences, termed nuclear localization sequences
       (NLSs). The NLS is recognized by cytoplasmic NLS-recep-
       tors that dock at the nuclear pore complex which subse-
       quently catalyzes the translocation across the nuclear
       envelope. Additional soluble factors are required for
25   these transport reactions.
              The nuclear pore complex mediates also diffu-
       sion, and proteins of molecular masses of 40kD or less
       can enter the nucleus via passive diffusion. By
       contrast, proteins that exceed the size of the diffu-
30   sion channel enter the nucleus by a facilitated process
       that requires energy and a NLS. Polypeptides larger
       than 70kD are excluded from nuclei if they do not carry
       a functional NLS. NLSs can be divided into several
       classes. The NLS present in SV40 T-antigen is an exam-
35   ple for a "simple" NLS containing a stretch of basic
       amino acid residues. A second class of NLSs contains
       "bipartite" sequences that carry two clusters of posi-

tively charged amino acid residues that are separated by a spacer region, such as the NLS of *Xenopus laevis* nucleoplasmin. Another type of targeting sequences includes complex NLSs which do not fit into the classes

5   of simple or bipartite signals.

So far, most experimental systems for the analysis of protein import in higher eukaryotes were based on *in vitro* reconstitution of transport or the injection of nuclear substrates into single cells. These

10   experiments are tedious and time-consuming. A caveat of some of the reporter proteins used in these studies is their post-translational modification such as phosphorylation which can modulate nuclear traffic.

It would be highly desirable to be provided with

15   improved tools to monitor nuclear transport of proteins *in vivo* and *in vitro*.

SUMMARY OF THE INVENTION

For the purpose of the present invention the

20   following abbreviations are defined below.

ADPP	ala-asp-pro-pro
APGP	ala-pro-gly-pro
BSA	bovine serum albumin
DAPI	4',6'-diamidino-2-phenylindole
FACS	Fluorescence activated cell sorting
GFP	*Aequorea victoria* green fluorescent protein
GNSP	gly-asn-ser-pro
NLS	nuclear localization sequence
NP-NLS	NLS of *Xenopus* laevis nucleoplasmin
PBS	phosphate buffered saline
pKS$^+$	plasmid pBluescript KS$^+$
SV40-NLS	NLS of SV40 T-antigen
SV40*-NLS	mutant form of SV40-NLS
SV40inv-NLS	inverse sequence of SV40-NLS.

(line numbers: 25 beside FACS, 30 beside NP-NLS, 35 beside SV40inv-NLS)

To analyze nucleocytoplasmic traffic in growing cells, an experimental system which is based on the inducible synthesis of fluorescent reporter proteins was developed. Reporter proteins of various sizes described here carry different NLSs which are fused to the *Aequorea victoria* green fluorescent protein (GFP). These substrates were expressed and localized in mammalian culture cells. In addition to the analysis of facilitated protein transport into nuclei, assays to study passive diffusion from the nucleus into the cytoplasm were described. To facilitate the generation of gene fusions that encode different mutants of GFP with or without a nuclear localization sequence several new cloning vectors were generated.

Moreover, in accordance with the present invention, a stable cell line that produces a nuclear reporter protein under control of an inducible promoter was analyzed. Upon induction, single cells of this clone synthesize similar amounts of the reporter protein. In accordance with the present invention, such cell lines offer the advantage that large numbers of cells can be tested simultaneously under identical conditions. Additional stable cell lines are currently generated for more detailed analyses of transport and diffusion across the nuclear envelope. Taken together, novel experimental tools to investigate nucleocytoplasmic transport and diffusion in intact mammalian cells were described.

In addition to the analysis of nuclear traffic in growing cells, tagged nuclear transport substrates carrying various numbers of GFP to study nucleocytoplasmic traffic *in vitro* were also generated. These substrates can be expressed in *Escherichia coli* and purified by metal affinity-chromatography. The purified substrates are currently tested with semi-permeabilized

HeLa cells and with yeast spheroplasts to demonstrate that these proteins are bona fide substrates for nuclear transport.

In accordance with the present invention there
5   is provided genes encoding reporter proteins to monitor transport and passive diffusion of proteins across the nuclear envelope in mammalian cells, which comprises a protein carrying a nuclear localization sequence (NLS) fused to at least one copy of a reporter protein,
10   wherein the reporter protein allows for the visualization of the reporter fusion protein.

In accordance with one embodiment of the present invention, the NLS derived from SV40 T-antigen is connected to the reporter protein via a linker sequence
15   encoding small amino acid residues. The NLS may also be derived from *Xenopus laevis* nucleoplasmin or present in the C-terminal portion of nucleoplasmin.

In accordance with one embodiment of the present invention, there is provided an expression vector to
20   transiently and stably express reporter genes of the present invention, which comprises a gene encoding a protein carrying a nuclear localization sequence (NLS) fused to at least one copy of a reporter protein, wherein expression of the gene is under the control of
25   an inducible promoter.

The NLS may be connected to the reporter protein via a linker sequence encoding small amino acid residues.

In accordance with another embodiment of the
30   present invention, the reporter protein may be any fluorescent protein such as *Aequorea victoria* green fluorescent protein (GFP).

In accordance with one embodiment of the present invention, there is provided, a cell line stably
35   expressing a nuclear reporter protein to monitor trans-

port and passive diffusion of proteins across the
nuclear envelope, which comprises a cell line trans-
fected by the expression vector of the present inven-
tion.

5   The NLS may be fused to the reporter protein by
a linker encoding small amino acid residue.

The cell lines may be selected from the group
consisting of HeLa, COS and CHO.

In accordance with another embodiment of the
10   present invention, there is provided an *in vivo* assay
for screening compounds which affect transport and/or
passive diffusion of proteins across the nuclear enve-
lope in mammalian cells, which comprises the steps of:

a)   treating a stably transfected cell line of the
15   present invention with a compound; and

b)   visualizing the distribution of reporter protein
in nuclei and cytoplasm.

In accordance with another embodiment of the
present invention, there is provided an *in vitro* assay
20   for screening compounds which affect transport and/or
passive diffusion of proteins across the nuclear enve-
lope in mammalian cells, which comprises the steps of:

a)   treating semi-permeabilized cells which support
in vitro nuclear transport with a compound and a pro-
25   tein of the present invention; and

b)   visualizing the distribution of reporter protein
in nuclei and cytoplasm.

In accordance with one embodiment of the present
invention, the cells may be treated with the compound
30   and protein concurrently or one after the other.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates a schematic representation of
different fluorescent reporter proteins containing GFP
35   used in this study;

Fig. 2 illustrates transient expression of fusion proteins harboring one copy of GFP;

Fig. 3 illustrates transient expression of transport substrates with two and four copies of GFP;

5   Fig. 4 illustrates the effect of linker sequences on the localization of SV40-GFP4;

Fig. 5 illustrates the localization of reporter proteins harboring wild type and mutant versions of SV40-NLS;

10   Figs. 6A and 6B illustrate nucleocytoplasmic diffusion of NP-GFP, (A) chilling of cells, (B) treatment with sodium azide/deoxyglucose at different temperatures;

Fig. 7 illustrates the kinetics of the diffusion 15 of NP-GFP across the nuclear envelope in HeLa cells treated with sodium azide/deoxyglucose; and

Fig. 8 illustrates stably transfected HeLa cells expressing nucleoplasmin-GFP.

20 **DETAILED DESCRIPTION OF THE INVENTION**

In accordance with the present invention, there is provided an experimental system to study nucleocytoplasmic traffic of proteins in living mammalian cells. Towards this goal, substrates were generated that 25 contain one or more copies of *Aequorea victoria* green fluorescent protein (GFP). Reporter proteins of various sizes that carry different nuclear localization sequences (NLSs) were created to follow facilitated transport and passive diffusion across the nuclear 30 envelope. The expression of reporter genes was controlled by an inducible promoter. Transiently and stably transfected HeLa cells were employed to follow the sorting of fluorescent reporter proteins. When NLS-GFP fusions were located in HeLa cells it was found 35 that direct fusion of the NLS derived from SV40 T-anti-

gen to GFP prevented nuclear accumulation of the protein. However, insertion between NLS and GFP of different linkers encoding small amino acid residues produced reporter proteins that were competent for

5   nuclear import.

Furthermore, it is demonstrated in accordance with the present invention that a small protein of approximately 30kD that harbors one copy of GFP and the bipartite *Xenopus* nucleoplasmin NLS accumulates effi-

10   ciently in nuclei of HeLa cells. Under conditions which abolish active facilitated nuclear transport the protein diffuses across the nuclear envelope. This polypeptide can therefore be used as a reporter to analyze passive diffusion between nucleus and cyto-

15   plasm.

In addition to transient expression of novel transport substrates, stable cell lines that carry different genes for a fluorescent nuclear reporter protein were created. These cell lines provide an

20   unique and novel tool for the characterization of nuclear protein import and diffusion in dividing human cells.

Based on the substrates to study nuclear traffic in growing cells of the present invention, histidine

25   tagged versions of reporter proteins which permits their purification by metal affinity chromatography were also generated.

EXPERIMENTAL PROCEDURES

30   **Cell culture and transfection**

HtTA-1 cells are described in detail in Damke et al., 1995 (Damke, H. et al., 1995, *Meth. Enzym.*, **257**:209-220). HtTA-1 cells contain the tTA-element which permits the control of gene expression by addi-

35   tion of tetracycline to the growth medium.

HeLa cells were grown in Dulbecco's modified essential medium (DMEM) containing penicillin G (50U/ml), streptomycin (50µg/ml) and 8 % fetal bovine serum (complete medium). Cultures were maintained at

5   37°C in a humidified atmosphere containing 5% CO_2. For transient transfections cells were subcultured on six-well tissue culture plates and DNA was introduced with the calcium phosphate/DNA coprecipitation method and CalPhos Maximizer™ (Bio/Can, Mississauga, Ontario) or

10 by use of the DOSPER™ Liposomal Transfection Reagent (Boehringer Mannheim, Canada) according to the manufacturer's instructions. Approximately 16 hrs to 24 hrs after addition of DNA, medium was removed and HeLa cells were kept in complete medium containing 50 nM

15 dexamethasone. To generate stable cell lines, HtTA-1 cells were transfected following the modified transfection protocol described (Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989, in *Molecular Cloning*, 2nd ed., pp 16.33-16.36, Cold Spring Harbor Laboratory Press).

20
Detection of transiently transfected cells expressing GFP-derivatives

Cells were incubated overnight with DNA followed by addition of fresh medium. Approximately 7 hours

25 after the change of medium, cells were harvested by trypsinization and transferred to LabTek tissue culture chamber slides (Gibco, BRL). HeLa cells were kept for another 16 to 44 hours in complete medium containing 50 nM dexamethasone. To study diffusion, cells were washed

30 with medium lacking dexamethasone and incubated in dexamethasone-free medium for 1 hour at   37°C prior to analysis of diffusion. To follow diffusion of nuclear substrates across the nuclear envelope, cells were washed with PBS and incubated in PBS in the cold.

35 Alternatively, cells were treated with 10 mM sodium

azide and 10 mM 2-deoxy D-glucose in PBS as described
for Figs. 6 and 7.

All subsequent steps were carried out at room
temperature. For fixation slides were washed twice in
5   phosphate buffered saline (PBS), incubated for 25 min
in PBS/3.7% formaldehyde and washed three times with
PBS. Samples were incubated with PBS containing 4',6'-
Diamidino-2-phenylindole (DAPI) and 2 mg/ml bovine
serum albumin (BSA), and washed twice in PBS. Slides
10   were mounted in 30% glycerol in PBS. Slides were sealed
with rubber cement and kept at 4°C. The fluorescence
signals were stable for several weeks.

Generation of stable cell lines and detection of
15   **nuclear reporter proteins**
HtTA-1 cells were grown and transfected as
described above. Transfected cells were grown under
standard conditions for at least two weeks without
selection. One day after addition of 50 nM dexametha-
20   sone, cells were sorted by FACS into 24-well plates
under sterile conditions. A maximum of one cell per
well was obtained after sorting. Cells were further
grown for two to three weeks and analyzed for the syn-
thesis of nuclear reporter proteins. Towards this end,
25   stable cell lines were kept in the presence of 50 nM
dexamethasone (2 days) and analyzed by fluorescence
microscopy as described above.
Plasmid constructions
Plasmid pGRE5-2, was used as a vector for genes
30   encoding different fluorescent substrates. Gene expres-
sion is controlled by glucocorticoid response elements
and can be induced by dexamethasone (Mader, S and
White, J., 1993, *Proc. Natl. Acad. Sci USA*, **90**:5603-
5607). A mutant version of GFP in pBluescriptKS$^+$ carry-
35   ing a Ser65 --> Thr65 substitution by PCR-amplification
of the GFP-DNA using appropriate primers was generated.

This created plasmid p580 which contains the mutant GFP allele cloned into the KpnI/EcoRI sites of pBluescript KS$^+$ (Chatterjee, S. and Stochaj, U., 1996, *BioTechniques*, **21**:62-63).

Plasmids encoding nuclear substrates with one or two copies of GFP

The generation of the gene encoding Nucleoplasmin-GFP, i.e., the C-terminal half of *Xenopus* nucleoplasmin fused to a single copy of GFP has been described (Chatterjee, S. and Stochaj, U., 1996, *BioTechniques*, **21**: 62-63). A gene fusion encoding nucleoplasmin NLS and GFP, termed NP-GFP, was created by fusion of oligonucleotides encoding the NLS (Barth, W. and Stochaj, U., 1996, *Biochem. Cell Biol.*, **74**: 363-372) to GFP. Plasmid p709 is derived from pGRE5-2 and codes for NP-GFP. Gene fusions containing two copies of GFP were created by removal of the UAG stop codon of GFP followed by insertion of a NotI linker (12mer, New England Biolabs). In addition, a NotI-linker was inserted into the KpnI-site of plasmid p580. Both copies of the GFP coding sequence were fused in frame via their NotI-sites thereby creating GFP2 in pBluescript (plasmid p690). Insertion of the KpnI/EcoRI fragment of p690 into the KpnI/EcoRI sites of pGRE5-2 created plasmid p785. For p785, the expression of GFP2 is driven by an inducible promoter carrying 5 glucocorticoid response elements.

Plasmids encoding fusion proteins containing four copies of GFP

DNAs encoding different NLSs were cloned into pGRE5-2. These vectors were digested with XhoI and EcoRI and a 2.8 kb XhoI/EcoRI fragment encoding four copies of GFP was ligated into the vector DNA. Following this ligation, DNA was digested with XhoI and AgeI. Recessed ends were filled in and DNA was autoli-

gated. The resulting plasmids p732, p733, p734 and p767 encode fusion proteins NP-GFP4, SV40-GFP4, SV40*-GFP4, and SV40inv-GFP4, respectively (Fig. 1). Derivatives of plasmids carrying wild type or mutant versions of SV40-

5 NLS fused to GFP4 were generated by in frame insertion of linker sequences. Constructs encoding a direct fusion of NLS and GFP were digested with AgeI, incubated with T4-polymerase in the presence of 4 dNTPs. 8mer linkers (New England Biolabs) carrying a BamHI,

10 EcoRI or SmaI-site were inserted. Linker insertion generated the following additional amino acid residues between NLS and the first copy of GFP: BamHI ala-asp-pro-pro (ADPP), EcoRI gly-asn-ser-pro (GNSP), SmaI ala-pro-gly-pro (APGP). Protein SV40-GFP4 that contains the

15 linker sequence ala-asp-pro-pro is referred to as SV40-ADPP-GFP4. All other constructs are named accordingly. Plasmid p713 encodes GFP4 whose expression is controlled by five glucocorticoid response elements. To obtain this plasmid a KpnI/EcoRI fragment coding for

20 the in frame fusion of four copies of GFP was inserted into the KpnI/EcoRI sites of pGRE5-2.

Plasmids to generate gene fusions containing GFP or NP-GFP

25 To facilitate the generation of gene fusions containing different forms of GFP the following constructs were generated: Plasmid p827 was obtained by inserting a XbaI/PstI fragment encoding a mutant GFP (L64 T65) into the XbaI/PstI sites of pKS$^+$.

30 p657 is derived from p580; p580 was digested with BsmI and treated with T4-DNA-polymerase in the presence of 4 dNTPs. A NotI-linker (12mer, New England Biolabs) was ligated to the filled in DNA, followed by digestion with NotI and autoligation. p657 is used to

35 create fusion genes that encode GFP at their 5'-end. A derivative of p657 was generated by inserting the

NcoI/HpaI fragment of p827 into p657 that was treated
with NcoI and HpaI. The resulting plasmid p833 contains
GFP (L64 T65) carrying a NotI-linker at the former
BsmI-site.

5   Similarly, p834 was created by insertion of the
NcoI/HpaI fragment of p827 into plasmid p580.

Plasmids encoding histidine-tagged versions of fluorescent marker proteins

10   To generate genes encoding histidine-tagged
marker proteins, coding sequences for the various fluorescent substrates were inserted in the appropriate pQE
vector (Qiagen) to create in frame fusions of the
histidine tag and the reporter protein. Specifically,
15 to obtain His6-GFP4, plasmid p713 was digested with
AgeI and EcoRI, blunted and inserted into pQE11 which
was treated with BamHI and Klenow polymerase. These
manipulations created plasmid p779 which encodes His6-
GFP4 under control of the *Escherichia coli* tac pro-
20 moter. Plasmid p779 was digested with NotI, the 4.8kb
fragment was autoligated resulting in plasmid p788
which encodes His6-GFP2. Treatment of plasmid p779 with
HpaI and autoligation of the 4.1kb linearized band
generated plasmid p790 which carries the coding
25 sequence for His6-GFP1. To generate His6-NP-GFP4, a
HindIII/EcoRI fragment containing the coding sequence
for NP-GFP4 was blunted and cloned into pQE10 treated
with BamHI and Klenow-polymerase to remove recessive
ends. Plasmid p778 codes for protein His6-NP-GFP4.
30 Treatment of p778 with NotI and autoligation of the
4.8kb band generated plasmid p791 which encodes His6-
NP-GFP2. Digestion of plasmid p778 with HpaI and
autoligation of the 4.1kb band results in plasmid p792
which codes for His6-NP-GFP. To create histidine-tagged
35 versions of GFP4 derivatives carrying different SV40-
NLSs, the respective pGRE5-2 vectors containing the

constructs described were digested with EcoRI and
HindIII followed by treatment with T4 DNA-polymerase in
the presence of 4 dNTPs. Purified 2.8kb fragments were
inserted in pQE10 which had been treated with BamHI and
5   T4 DNA-polymerase plus 4 dNTPs. Plasmids p778, p791,
p792, p799, p788, p790, p818, p819, p824, p828, p829
and p830 and the different NLSs they encode are listed
in Table 1.

The different sequences listed in Table 1 are
10   described in Barth and Stochaj, 1996 (Barth, W. and
Stochaj, U., 1996, *Biochem. Cell Biol.*, **74**:363-372).
Inserted linker sequences BamHI, EcoRI and SmaI are
shown in italics (Table 1). Plasmids p827, p833 and
p834 carry a mutant GFP (F64L S65T) as described by
15   Cormack et al., 1996 (Cormack, B.P. et al., 1996, *Gene*,
174: 33-38). The backbones of the plasmids and the
respective fusion genes are listed in Table 1. In addi-
tion, it is stated which organisms can be used for the
inducible expression of gene fusions.

20

Table 1

Generation of different plasmids to analyze nuclear
transport of proteins and diffusion across the nuclear
25   envelope

Plasmid name	Backbone	Gene encoded	Inducible expression in
p580	pBluescript KS+	GFP	---
P585	pGRE5-2	Nucleoplasmin-GFP	mammalian cells
p657	pBluescript KS+	GFP carrying a NotI-linker at 3'-end	---
p827	pBluescript KS+	GFP L64 T65	---
p833	pBluescript KS+	same as p657 but GFP L64 T65	---
p834	pBluescript KS+	same as p657 but GFP L64 T65	---
P709	pGRE5-2	NP-GFP	mammalian cells

p775	pBluescript KS+	NP-GFP	---
p776	pBluescript KS+	NP-GFP (blue GFP)	---
p777	pGRE5-2	NP-GFP (blue GFP)	mammalian cells
p732	pGRE5-2	NP-GFP4	mammalian cells
p733	pGRE5-2	SV40-GFP4	mammalian cells
p734	pGRE5-2	SV40*-GFP4	mammalian cells
p767	pGRE5-2	SV40inv-GFP4	mammalian cells
p764	pGRE5-2	SV40-SmaI -GFP4	mammalian cells
p765	pGRE5-22	SV40-EcoRI-GFP4	mammalian cells
p795	pGRE5-2	SV40-BamHI-GFP4	mammalian cells
p780	pGRE5-2	SV40inv-BamHI-GFP4	mammalian cells
p781	pGRE5-2	SV40*-BamHI-GFP4	mammalian cells
p782	pGRE5-2	SV40inv-SmaI-GFP4	mammalian cells
p783	pGRE5-2	SV40*-SmaI-GFP4	mammalian cells
p784	pGRE5-2	SV40inv-EcoRI-GFP4	mammalian cells
p785	pGRE5-2	GFP2	mammalian cells
p778	pQE	His6-NP-GFP4	Escherichia coli
p791	pQE	His6-NP-GFP2	Escherichia coli
p792	pQE	His6-NP-GFP	Escherichia coli
p779	pQE	His6-GFP4	Escherichia coli
p788	pQE	His6-GFP2	Escherichia coli
p790	pQE	His6-GFP	Escherichia coli
p818	pQE	His6-SV40-BamHI-GFP4	Escherichia coli
p819	pQE	His6-SV40inv-BamHI-GFP4	Escherichia coli
p824	pQE	His6-SV40*-BamHI-GFP4	Escherichia coli
p828	pQE	His6-SV40-SmaI-GFP4	Escherichia coli
p829	pQE	His6-SV40*-SmaI-GFP4	Escherichia coli
p830	pQE	His6-SV40inv-SmaI-GFP4	Escherichia coli

Plasmids encoding different mutants of GFP

In accordance with the present invention, additional plasmids which are identical to those listed in

Table 1 but carry a different version of GFP are cur-
rently being generated. GFP used to generate these
plasmids carries a mutation at position at amino acid
residue 64. The wild type Phe64 was replaced by Leu64
5   (F64L). This mutant GFP-derivative GFPmut1, referred to
as GFP L64 T65, was obtained from B. R. Cormack and
published in 1996 (*Gene*, **174**:33-38). In addition,
nuclear substrates carrying the blue version of GFP are
being created. The blue version of GFP is mutant P4
10 obtained from Roger Tsien (Heim, R. et al., 1994, *Proc.
Natl. Acad. Sci.*, USA, **91**:12501-12504).

Plasmids for expression in *Saccharomyces cerevisiae*

Genes encoding different nuclear substrates
described in Table 1 are presently cloned into yeast
15 expression vectors. The expression of gene fusions in
these vectors is driven by the *GAL1* control region.
Gene expression is therefore induced when cells are
grown on galactose as carbon source.

20 <u>USE OF THE INVENTION</u>

Generation of nuclear substrates containing GFP

Different genes encoding fusion proteins that
are substrates for nuclear transport were created. The
various constructs used in our studies are depicted in
25 Fig. 1. Controls include proteins with one, two and
four copies of GFP, which are termed GFP, GFP2 and
GFP4, respectively. Fusion proteins were generated that
carry the nucleoplasmin bipartite NLS (NP), the C-
terminal half of nucleoplasmin (nucleoplasmin), SV40
30 wild type NLS (SV40), a mutant derivative (SV40*) and
the inverse signal (SV40inv). Additional fusions were
created that contain linker sequences between the NLS
and the first copy of GFP. Only one construct harboring
a linker sequence L and SV40-NLS is shown as an
35 example. The different linker sequences inserted and

fusion proteins containing these linkers are described below.

Control proteins include GFP and a fusion protein containing two and four copies of GFP, termed
5 GFP2 and GFP4, respectively. Polypeptide Nucleoplasmin-GFP contains the C-terminal half of *Xenopus* nucleoplasmin including the bipartite NLS and the DNA-binding region. Proteins SV40-GFP4, SV40*-GFP4, SV40inv-GFP4 and NP-GFP4 carry wild type or mutant
10 forms of the NLS at their N-termini as indicated. Derivatives of the proteins containing different linker sequences between NLS and GFP4 are described above. The estimated molecular masses of GFP2 is about 60kD, for GFP4 and the NLS-containing derivatives approximately
15 120kD. When synthesized in *E. coli* and analyzed by denaturing gels, proteins migrate as expected.

Transient expression of GFP-containing transport substrates in HeLa cells

20 To determine the cellular localization of trans-port substrates, HeLa cells were transiently trans-fected with plasmids that express fusion proteins under the control of an inducible promoter. Gene expression was induced by addition of dexamethasone to the growth
25 medium. As shown in Fig. 2 for plasmids encoding NP-GFP and GFP, in the presence of dexamethasone cells exhibit bright fluorescence. HeLa cells were transiently trans-fected with plasmids encoding NP-GFP (panels A, B) or GFP (panels C, D) and gene expression was induced with
30 dexamethasone as described herein. DAPI-staining of the DNA (panels A, C) and green fluorescence (panels B, D) is shown. NP-GFP is efficiently targeted to the nucleus due to the presence of the bipartite NLS derived from *Xenopus* nucleoplasmin. By contrast, non-transfected
35 cells display only a low level of autofluorescence (Fig. 2). Low autofluorescence signals were also

obtained for non-induced cells, i.e., cells kept in the absence of dexamethasone.

When GFP lacking a NLS was synthesized in HeLa cells, GFP was detected in both the cytoplasm and the

5   nucleus (Fig. 2). This distribution is expected, since the small size of the protein permits its translocation across the nuclear envelope by passive diffusion. We also expressed a gene encoding two copies of GFP (GFP2) in HeLa cells. GFP2, a protein with a molecular mass of

10   approximately 60kD was also found to traverse the nuclear envelope (Fig. 3). HeLa cells synthesizing GFP2 (panels A, B), GFP4 (panels C, D) and NP-GFP4 (panels E, F) were transiently transfected and processed in accordance with the present invention. DAPI-staining

15   (panels A, C, E) and green fluorescence (panels B, D, F) are illustrated. The amount of GFP2 in nuclei, however, was relatively low, most likely the larger molecular mass restricted its diffusion across the nuclear pore.

20   The nuclear pore mediates transport of macro-molecules across the nuclear envelope and molecules with a molecular mass of more than 70kD are excluded from the nucleus unless they carry a NLS. To obtain reporter proteins that exceed the size for passive

25   diffusion, we have generated substrates that contain four copies of GFP. As shown in Fig. 3, in transiently transfected cells GFP4 was restricted to the cytoplasm and could not be detected in nuclei (panels C, D). On the basis of these results we created several different

30   reporter proteins that contain four copies of GFP in addition to wild type or mutant forms of NLSs (Fig. 1).

Our initial experiments included the generation of constructs for which the NLS was directly fused to the first copy of GFP. For the bipartite nucleoplasmin-

35   NLS, i.e., NP-GFP4, efficient import into nuclei was

obtained (Fig. 3, panels E, F). By contrast, constructs
containing wild type SV40-NLS fused to GFP4 did not
accumulate in nuclei (Fig. 4, panels A and B).
Similarly, reporter proteins containing mutant versions

5   of SV40-NLS fused to GFP4 were excluded from nuclei. It
has previously been reported for SV40-NLS and other
NLSs that flanking protein sequences, i.e., context of
the NLS can affect the efficiency of nuclear targeting.
We therefore reasoned that addition of amino acid resi-

10   dues between SV40-NLS and GFP4 could improve nuclear
targeting of the fusion protein. Different linker
sequences encoding short stretches of amino acid resi-
dues were inserted between SV40-NLS and GFP4. Linker
sequences coded for proline and amino acid residues

15   with small side chains that have the potential to form
β-turns. Residues with small side chains are expected
to provide flexibility of the linker. The linkers used
generated the following extra amino acid sequences (one
letter code) BamHI - ADPP, EcoRI - GNSP and SmaI-APGP.

20   Reporter proteins containing wild type SV40-NLS and one
of the different linker sequences were found to accumu-
late in nuclei (Fig. 4, panels C-H). Fusion proteins
SV40-GFP4 containing SV40-NLS directly fused to GFP
(panel A, B), or connected by different linker

25   sequences were localized. Insertion of the peptide
sequence ADPP (panels C, D), GNSP (panels E, F) and
APGP (panels G, H) resulted in nuclear localization of
reporter proteins. DAPI staining (panels A, C, E, G)
and green fluorescence (panels B, D, F, H) were

30   analyzed in parallel.
        This demonstrates that linker insertion gener-
ated altered nuclear substrates that are recognized by
the nuclear transport apparatus and targeted to the
nucleus. We concentrated on constructs containing link-

35   ers ADPP or APGP for further studies. Towards this end,

linker insertions were created for SV40*-GFP4 and
SV40inv-GFP4, and we analyzed the cellular localization
of the respective proteins (Fig. 5). GFP4-derivatives
containing the linker sequence ADPP (Panels A to F) or
5   APGP (panels G to L) were transiently synthesized in
HeLa cells. Substrate proteins contained wild type
SV40-NLS (panels A, B, G, H), mutant SV40*-NLS (panels
C, D, I, J) or inverse SV40inv (panels E, F, K, L).
Staining with DAPI (panels A, C, E, G, I, K) and green
10   fluorescence (panels B, D, F, H, J, L) is shown.

        Transport substrates carrying SV40inv-NLS were
excluded from the nucleus (Fig. 5). By contrast, pro-
teins containing the mutant SV40*-NLS were not excluded
from nuclei. However, these reporter proteins did not
15   accumulate in nuclei. These results are consistent with
previously published data that show nuclear targeting
via this mutant NLS albeit with reduced efficiency.

        Taken together, we have generated several
nuclear transport substrates containing four copies of
20   GFP and different NLSs. The type and context of the NLS
specifies the localization of the reporter protein that
can be easily followed in living cells.

Diffusion from the nucleus into the cytoplasm

        In order to study diffusion of a polypeptide
25   across the nuclear envelope, a reporter protein is
required that is small enough to traverse the nuclear
pore in the absence of nuclear import or export sig-
nals. NP-GFP, i.e., a fusion protein that contains the
Xenopus nucleoplasmin NLS fused to one copy of GFP
30   accumulated in nuclei under normal growth conditions
(Fig. 2). However, the small size of approximately 30kD
should permit NP-GFP to also traverse the nuclear pore
complex by passive diffusion. As described above, tran-
siently transfected cells synthesizing NP-GFP showed
35   bright nuclear fluorescence (Fig. 2). To test whether

this protein can diffuse across the nuclear envelope,
the inducer dexamethasone was removed and cells were
transferred to 4°C. Although this led to diffusion of
NP-GFP from the nucleus into the cytoplasm, even after
5   4 hours of incubation at 4°C the nuclear fluorescence
was more intense than the fluorescence signal seen for
the cytoplasm. If cells were incubated for 4 hours at 4
°C and returned to 37°C in growth medium cytoplasmic NP-
GFP was re-imported into nuclei demonstrating that
10   active facilitated transport was regained under normal
growth conditions. Prolonged incubation at 4°C resulted
in rounding of HeLa cells which was also reversible.
Round cells flattened again and spread on the slide
after overnight incubation at 37°C.

15   A more pronounced exit of NP-GFP from the
nucleus into the cytoplasm was obtained when cells were
incubated 2 hours at 4°C and subsequently kept for 2
hours on ice. Under these conditions fluorescence
signals observed for nuclei and for the cytoplasm were
20   of similar intensity, indicating the efficient equili-
bration of NP-GFP between nucleus and cytoplasm
(Fig. 6A). HeLa cells transiently expressing the NP-GFP
gene were incubated for 2 hours at 4°C, followed by 2
hours on ice. The distribution of fluorescence was
25   determined before (panels A, B) and after chilling
(panels C, D). After incubation on ice cells were
returned to 37°C and incubated overnight in the absence
of the inducer dexamethasone to test for re-import of
NP-GFP into the nucleus (panels E, F). Panels A, C, D
30   show phase contrast, panels B, D and F the distribution
of fluorescence.

         These results support the idea that in chilled
HeLa cells NP-GFP left the nucleus by diffusion and
nuclear accumulation of NP-GFP is prevented due to the
35   absence of active facilitated transport into the

nucleus. To determine whether nuclear exit of NP-GFP is reversible, cells were first treated 2 hours at 4°C and 2 hours on ice and subsequently incubated in growth medium at 37°C (Fig. 6A). Under these conditions cyto-
5   plasmic NP-GFP was re-imported into nuclei demonstrating that diffusion across the nuclear envelope was reversible and cells recovered from chilling.

In addition to cold treatment, we also tested the effect of sodium azide/2-deoxy D-glucose
10 (azide/deoxyglucose) on the localization of NP-GFP. After incubation of transiently transfected cells with azide/deoxyglucose, NP-GFP equilibrated efficiently between nucleus and cytoplasm (Fig. 6B). Transiently transfected HeLa cells containing NP-GFP were incubated
15 with azide/deoxyglucose as described herein. The localization of NP-GFP was determined before (panels A, B and G, H) and after treatment with sodium azide/deoxyglucose for 45 min (panels C, D and I, J). Slides were washed in PBS and incubated overnight with
20 medium lacking dexamethasone to analyze accumulation of NP-GFP in the nucleus (panels E, F and K, L). Identical experiments were carried out on ice (panels A - F) and at room temperature (panels G - L). Phase contrast is presented in panels A, C, E, G, I, K, the location of
25 fluorescent proteins is shown in panels B, D, F, H, J and L.

We further determined whether incubation with azide/deoxyglucose led to nuclear exit of NP-GFP at other temperatures. As demonstrated in Fig. 6B treat-
30 ment at room temperature or at 37°C results in diffusion of NP-GFP across the nuclear envelope. For all the different temperatures tested, we found that upon return of cells to normal growth medium and 37°C, re-import of NP-GFP was observed for at least a fraction
35 of cells. After incubation with azide/deoxyglucose at

37°C fewer cells seemed to recover when compared to those treated at room temperature or incubated on ic .

To study diffusion of NP-GFP across the nuclear envelope in more detail, we followed the distribution

5 of fluorescence in cells that were incubated with azide/deoxyglucose at room temperature (Fig. 7). HeLa cells were incubated at room temperature with sodium azide/deoxyglucose and the nucleocytoplasmic distribution of the fluorescence signal was determined for 0

10 min, 10 min, 30 min, 45 min, 60 min and 90 min of the incubation with sodium azide/deoxyglucose as indicated in Fig. 7.

A gradual increase of cytoplasmic fluorescence was seen upon treatment with azide/deoxyglucose. The

15 equilibration of NP-GFP was complete at approximately 45 min, and prolonged incubations with inhibitors did not lead to further changes.

Generation and characterization of a stable cell line
20 **expressing Nucleoplasmin-GFP**

When induced with dexamethasone, transiently transfected cells show differences in the fluorescence which reflects different copy numbers of the plasmids introduced into each cell. In addition, transiently

25 transfected cells loose the plasmid after prolonged culturing. Therefore, for many studies it is advantageous to have a uniform population of cells with comparable fluorescence. To this end, we created a stable cell line which carries the gene for nucleoplasmin-GFP.

30 Cells were transfected and grown for two weeks without selection. Dexamethasone was added to the growth medium and cells displaying fluorescence were sorted by FACS. Under these conditions, approximately one in 10,000 cells gave a fluorescence signal. Single cells were

35 isolated and further cultured. Characterization of stable transfectants demonstrated that the fluorescence

signal observed varied between different clones. This
most likely reflects variable levels of gene expression
due to differences in the integration sites of the
plasmid. However, fluorescence signals for single cells
5   derived from the same clone were comparable (Fig. 8,
panels C, D). HeLa cells were transfected with a plas-
mid encoding Nucleoplasmin-GFP and stable transfectants
were selected as described herein. Green fluorescence
obtained after induction of gene expression with
10   dexamethasone (panel B) or in the absence of dexametha-
sone (panel D) was determined. Nuclei were detected by
staining with DAPI (panel A, C).

In the absence of the inducer dexamethasone, no
fluorescence signal was obtained demonstrating the
15   tight control of gene expression in this experimental
system (Fig. 8).

Additional stable cell lines have been generated
that express NP-GFP and GFP only. We are presently
creating a larger number of these stable cell lines to
20   obtain clones with improved fluorescence.

Previous and future use of plasmids and cell lines

We have generated a nuclear transport system
that permits the analysis of nuclear import and passive
25   diffusion of proteins from the nucleus into the
cytoplasm. To achieve this goal, we have created a
variety of fusion proteins that contain one, two or
four copies of GFP.

30   **Generation of fusion proteins containing GFP and a
nuclear localization signal**

Different plasmid vectors encoding NLS-GFP
derivatives will be useful to create fusion proteins
that are targeted to the nucleus. If the protein of
35   interest has deleterious effects, it is desirable to
have an experimental system that permits the controlled
induction of genes. To this end, promoters regulated by

steroid hormone binding sites as described here for
pGRE-derivatives will be optimal.

Analysis of nuclear transport in mammalian cell lines

5   Analysis of facilitated transport in HeLa cells
requires substrates that are unable to enter the
nucleus by diffusion. Therefore, we have designed
fusion proteins whose size prevents them from diffusing
across the nuclear pore. These nuclear substrates
10   contain four copies of GFP, and in the absence of a
functional NLS GFP4 was excluded from nuclei. By
contrast, protein GFP2 which contains two copies of GFP
was present in nuclei, indicating that GFP2 could dif-
fuse across the nuclear envelope. The translocation of
15   GFP2 into nuclei is not mediated by a cryptic NLS since
such a signal would also be present in GFP4, a protein
we have shown to be excluded from nuclei. Thus,
although the molecular mass of GFP2 is close to the
exclusion size of nuclear pores for passive diffusion,
20   it can still traverse the nuclear envelope of HeLa
cells.

        Since GFP4 remained cytoplasmic in HeLa cells,
we concentrated on the generation of reporter proteins
that harbor four copies of GFP. Nucleoplasmin NLS pro-
25   vided a functional signal when directly fused to the
GFP4-moiety, demonstrating that the context provided by
GFP4 permits the recognition of the bipartite signal
and nuclear accumulation of NP-GFP4. In contrast to
nuclear targeting via nucleoplasmin NLS, direct fusion
30   of SV40-NLS to GFP4 did not result in nuclear targeting
of the fusion protein. These results can be interpreted
as the failure of cytoplasmic factors to bind the sim-
ple SV40-NLS when fused to GFP4 due to steric
hindrance. Alternatively, the flanking sequences pro-
35   vided by GFP could interfere with its function. It is
currently believed that nuclear transport of proteins

harboring SV40-NLS and the bipartite nucleoplasmin-NLS
are both recognized by importin-alpha during nuclear
transport. Since nucleoplasmin NLS directly fused to
GFP or GFP4 was efficiently targeted to the nucleus, it
5   is reasonable to assume that the N-terminal sequence of
GFP does not inhibit binding to cytoplasmic NLS-recep-
tors in general. We therefore favor the interpretation
that SV40-NLS is not readily accessible for recognition
by nuclear transport factors when directly fused to
10   GFP4. Introduction of linker sequences that encode a
stretch of small amino acid residues, however, enabled
SV40-NLS to target GFP4 to the nucleus. Control experi-
ments were carried out with fusion proteins containing
identical linker insertions and SV40*-NLS or SV40inv-
15   NLS. SV40*-GFP4 did not accumulate and SV40inv-GFP4 was
excluded from nuclei demonstrating that the inserted
linker sequence does not function as a cryptic NLS.
When compared to GFP4 or SV40inv-GFP carrying different
linker insertions it was observed that SV40*-GFP was
20   not excluded from nuclei. This result is in agreement
with previous studies which showed that the mutant NLS
of SV40-T antigen harboring a Lys128->Thr128 mutation
can still function as a weak NLS.

25   **Analysis of diffusion across the nuclear envelope**
As previously described by us the small size of
GFP permits this protein to enter the nucleus by pas-
sive diffusion. Fusion of the bipartite nucleoplasmin
NLS to GFP efficiently mediated nuclear accumulation of
30   the reporter protein. The small size of NP-GFP, i.e.,
approximately 30kD, made it an ideal tool to study dif-
fusion from the nucleus into the cytoplasm.
For the experiments reported here, we demon-
strated that NP-GFP exited the nucleus at low tempera-
35   tures even in the absence of metabolic inhibitors.
Migration of NP-GFP into the cytoplasm of HeLa cells

during chilling is not caused by irreversible damage of the cells or the nuclear envelope. HeLa cells survived this treatment, and the nuclear substrate was re-imported into nuclei after shifting to normal growth conditions. The rapid nuclear exit of NP-GFP during chilling enables us to study diffusion in the absence of metabolic inhibitors which are likely to interfere with a variety of cellular processes.

In addition to the analyses described above, NP-GFP will be useful as a tool to test how various drugs might interfere with diffusion across the nuclear enve-lope. The possible effect by these agents can be easily followed by fluorescence microscopy.

Stable cell lines expression fluorescent reporter pro-tein

We also studied diffusion of the fluorescent reporter protein in the stable cell line synthesizing nucleoplasmin-GFP. However, we did not detect an equilibration of the fluorescence signal between nucleus and cytoplasm if cells were chilled or treated with sodium azide/deoxyglucose. Two possible hypotheses which are not mutually exclusive could explain this result: (i) The larger size of nucleoplasmin-GFP as compared to NP-GFP prevents its rapid diffusion across the nuclear envelope. (ii) Nucleoplasmin-GFP still con-tains the DNA-binding region of *Xenopus* nucleoplasmin, and binding to DNA could retain the reporter protein in the nucleus even in the absence of active facilitated transport across the nuclear membrane. This cell line will therefore be useful for studies that concentrate on the analysis of nuclear transport only.

Additional cell lines expressing NP-GFP or only GFP have also been generated, and we are currently creating additional cell lines which have improved fluorescence.

In general, all of the cell lines described will
allow the controlled expression not only of the fluo-
rescent reporter protein but also of additional genes
of interest. Since the cells contain the tTA-element
5   (Damke, H. et al., 1995, *Meth. Enzym.*, **257**:209-220),
they can be used to regulate the synthesis of a protein
of interest by addition of tetracycline to the growth
medium. Genes encoded on a tetracycline response plas-
mid are repressed in the presence of tetracycline,
10   whereas in the absence of tetracycline gene expression
is induced.

**Use of nuclear substrates in other mammalian cell lines
and in the yeast *S. cerevisiae***

15   Nuclear substrates described in this study were
also tested in other mammalian cell lines such as COS-7
and CHO cells. Similar results as described for HeLa
cells were obtained, indicating that the reporter
system we have developed is useful as a model to study
20   nuclear transport in different mammalian cells. Taken
together, we have established tools to study diffusion
and signal-mediated transport across the nuclear enve-
lope in higher eukaryotic cells. This sets the stage to
analyze nucleocytoplasmic traffic in living mammalian
25   cells in more detail.
           Expression of fluorescent reporter proteins
described here is currently also analyzed in the yeast
S. cerevisiae.

30   **Analysis of nuclear transport *in vitro***
           Histidine-tagged (His6) versions of fluorescent
reporter proteins have been created. Synthesis of these
proteins is inducible in *Escherichia coli*. Fusion
proteins are currently purified by affinity-purifica-
35   tion and analyzed in *in vitro* nuclear transport assays.
The advantage of these substrates is that they are easy
to purify in large quantities and at low costs.

Previously used substrates for *in vitro* studies involved the synthesis and covalent coupling of NLS-peptides, an expensive and laborious procedure.

While the invention has been described in con-
5 nection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any varia-tions, uses, or adaptations of the invention following, in general, the principles of the invention and
10 including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended
15 claims.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1.   Genes encoding reporter proteins to monitor transport and passive diffusion of proteins across the nuclear envelope in mammalian cells, which comprises a protein carrying a nuclear localization sequence (NLS) fused to at least one copy of a reporter protein, wherein the reporter protein allows for the visualization of the reporter fusion protein.

2.   The genes encoding proteins of claim 1, wherein the NLS is connected to the reporter protein via a linker sequence encoding small amino acid residues.

3.   The genes encoding proteins of claim 2, wherein the NLS is derived from *Xenopus laevis* nucleoplasmin or present in the C-terminal portion of nucleoplasmin.

4.   The genes encoding proteins of claim 3, wherein said reporter protein is a fluorescent protein.

5.   The genes encoding proteins of claim 4, wherein said fluorescent protein is *Aequorea victoria* green fluorescent protein (GFP).

6.   An expression vector to transiently and stably express reporter genes of claim 1, which comprises a gene encoding a protein carrying a nuclear localization sequence (NLS) fused to at least one copy of a reporter protein, wherein expression of the gene is under the control of an inducible promoter.

7.   The expression vector of claim 6, wherein the
NLS is connected to the reporter protein via a linker
sequence encoding small amino acid residues.

8.   The expression vector of claim 7, wherein the
reporter protein is a fluorescent protein.

9.   The expression vector of claim 8, wherein the
fluorescent protein is *Aequorea victoria* green
fluorescent protein (GFP).

10.   A cell line stably expressing a nuclear reporter
protein to monitor transport and passive diffusion of
proteins across the nuclear envelope, which comprises a
cell line transfected by the expression vector of claim
6.

11.   The cell line of claim 10, wherein NLS is fused
to reporter protein via a linker encoding small amino
acid residue.

12.   The cell line of claim 10, wherein said cell
line is selected from the group consisting of HeLa, COS
and CHO.

13.   An *in vivo* assay for screening compounds which
affect transport and/or passive diffusion of proteins
across the nuclear envelope in mammalian cells, which
comprises the steps of:
a)   treating a cell line of claim 10 with a
compound; and
b)   visualizing the distribution of reporter protein
in nuclei and cytoplasm.

14.   An *in vitro* assay for screening compounds which affect transport and/or passive diffusion of proteins across the nuclear envelope in mammalian cells, which comprises the steps of:

a)   treating semi-permeabilized cells which support *in vitro* nuclear transport with a compound and a protein of claim 1; and

b)   visualizing the distribution of reporter protein in nuclei and cytoplasm.

15.   The assay of claim 14, wherein said cells can be treated with the compound and protein concurrently or one after the other.

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FIG - 1

FIG. 2

Figure 3

FIG_4

5/9

0 min
cold

A

4 hours
cold

C

4 hours
cold,
ON
37⁰C

E

$FIG. 6A$
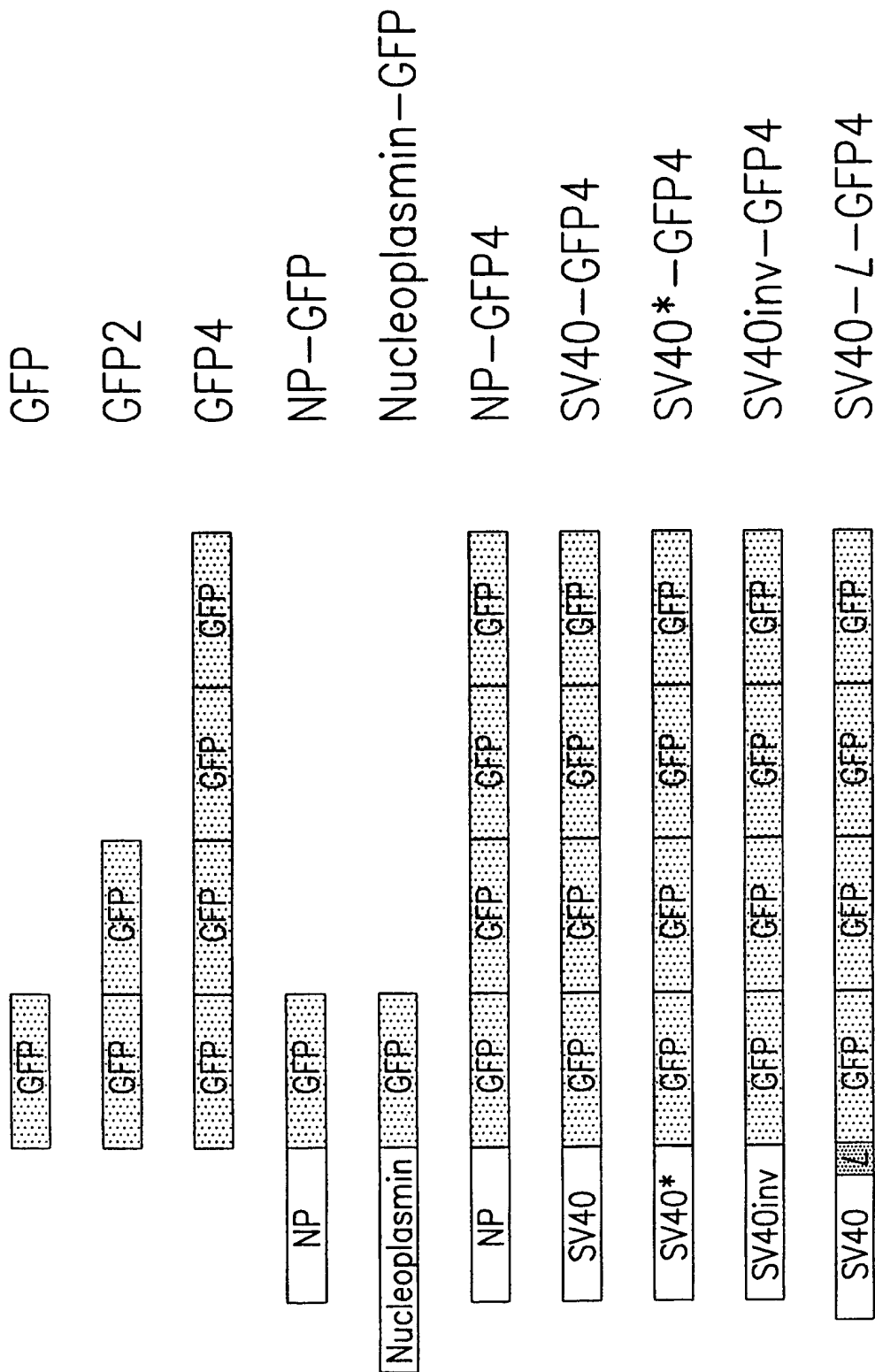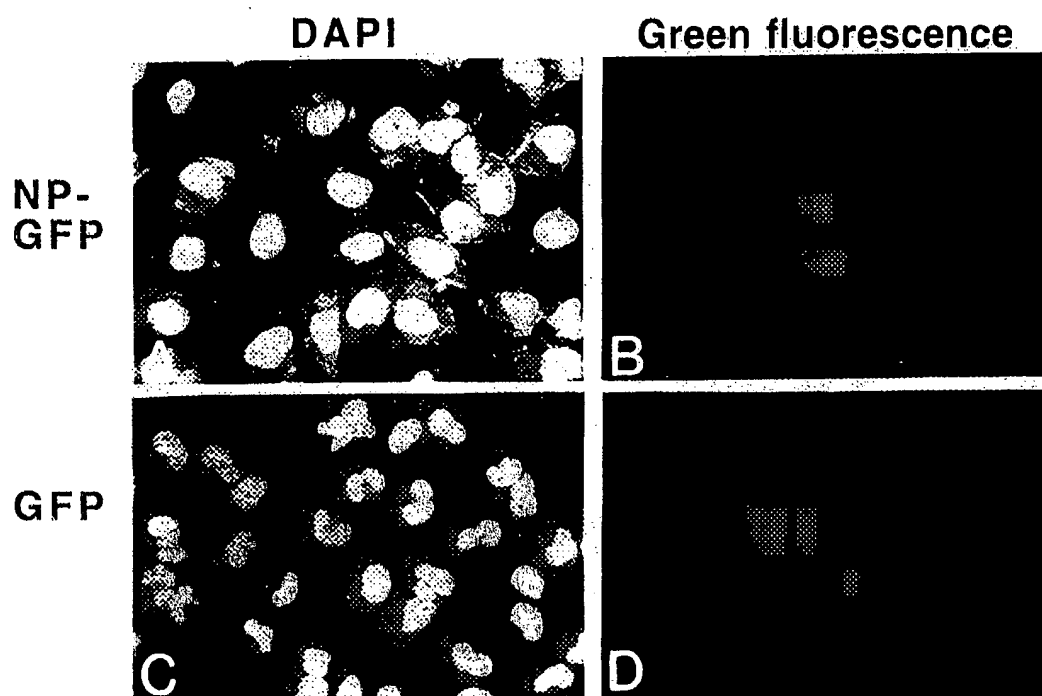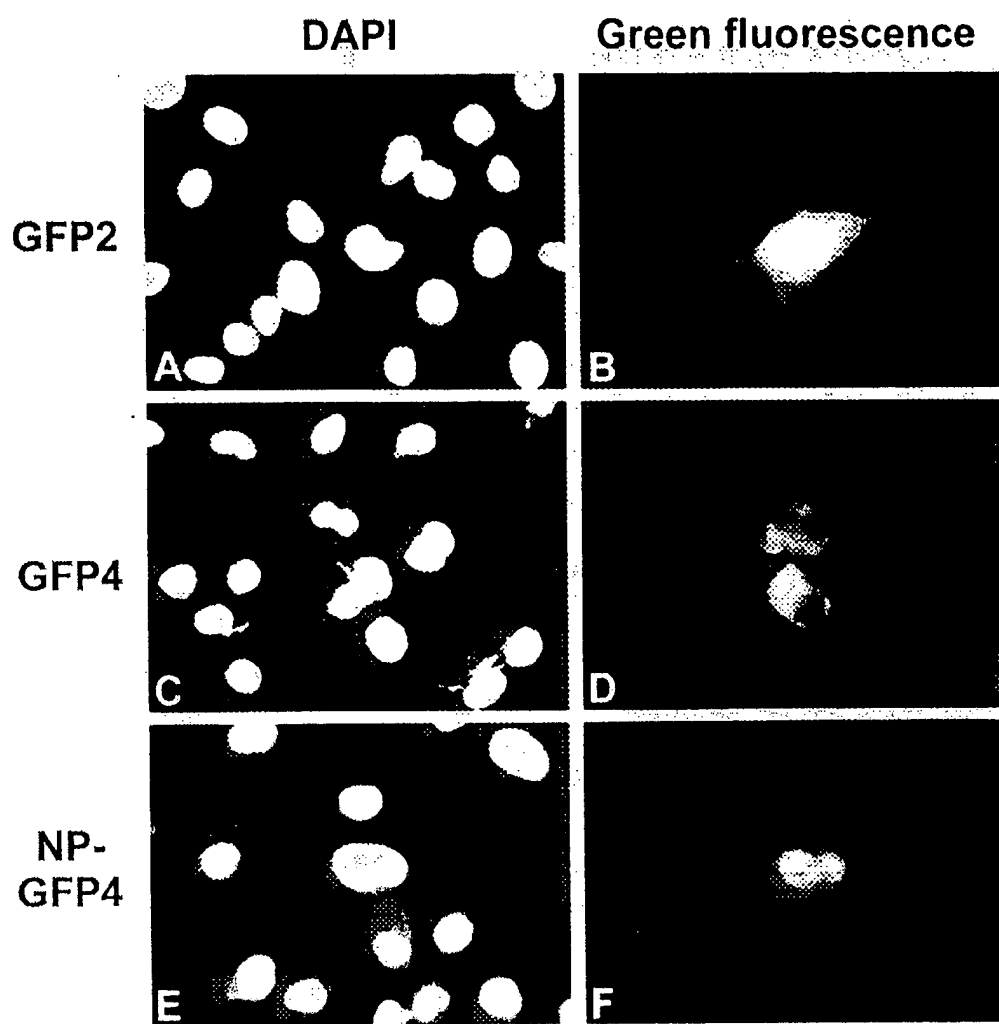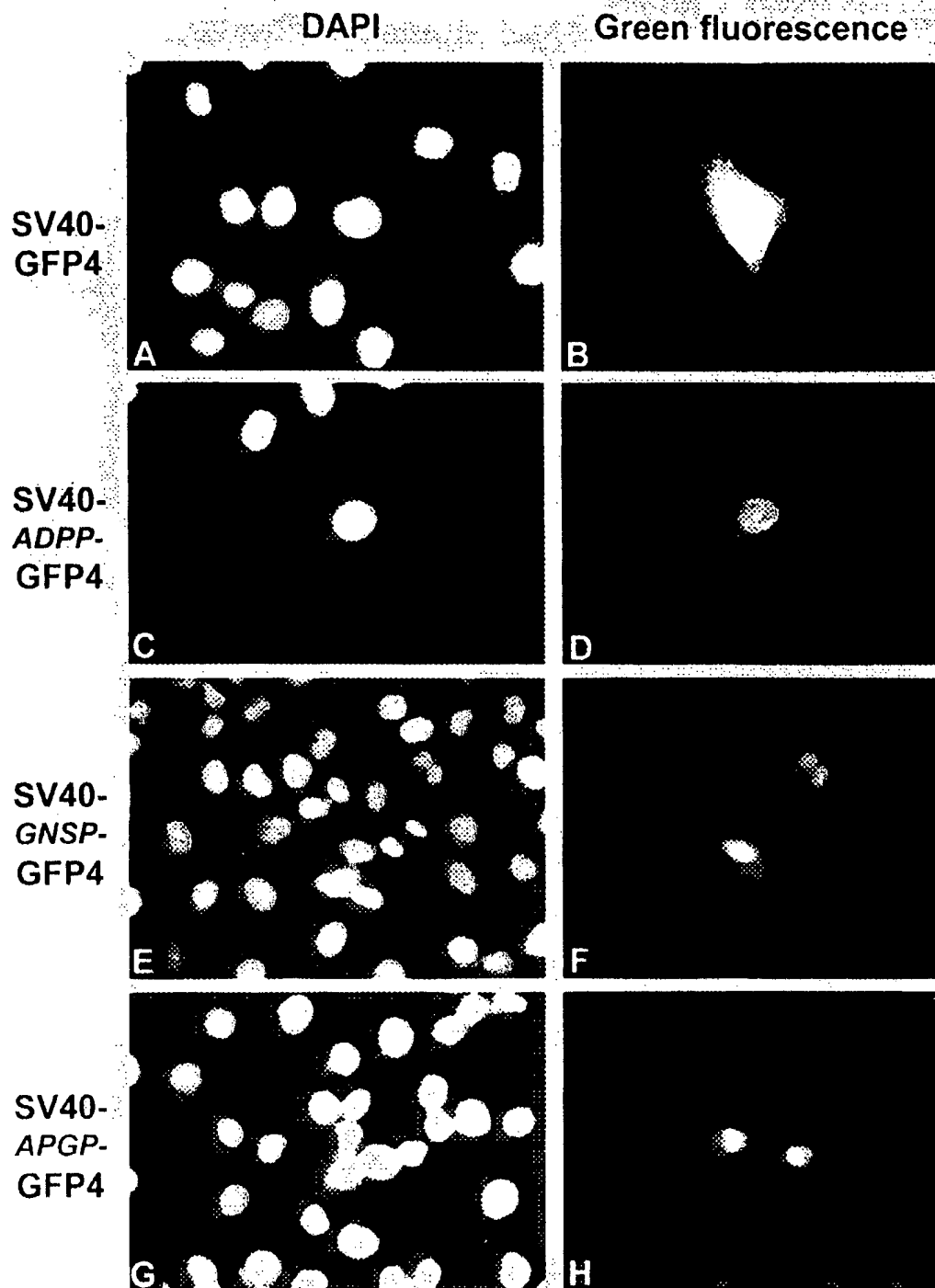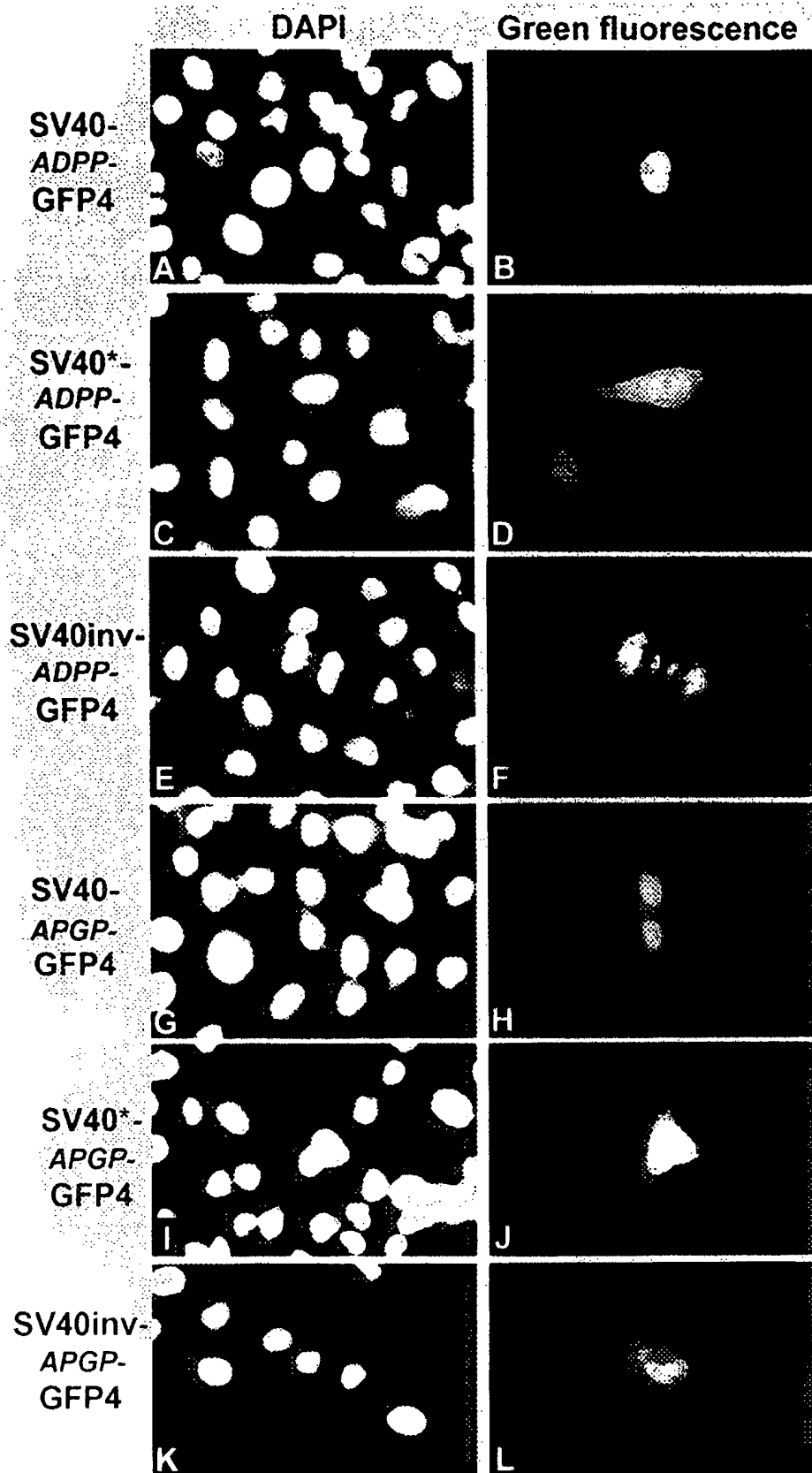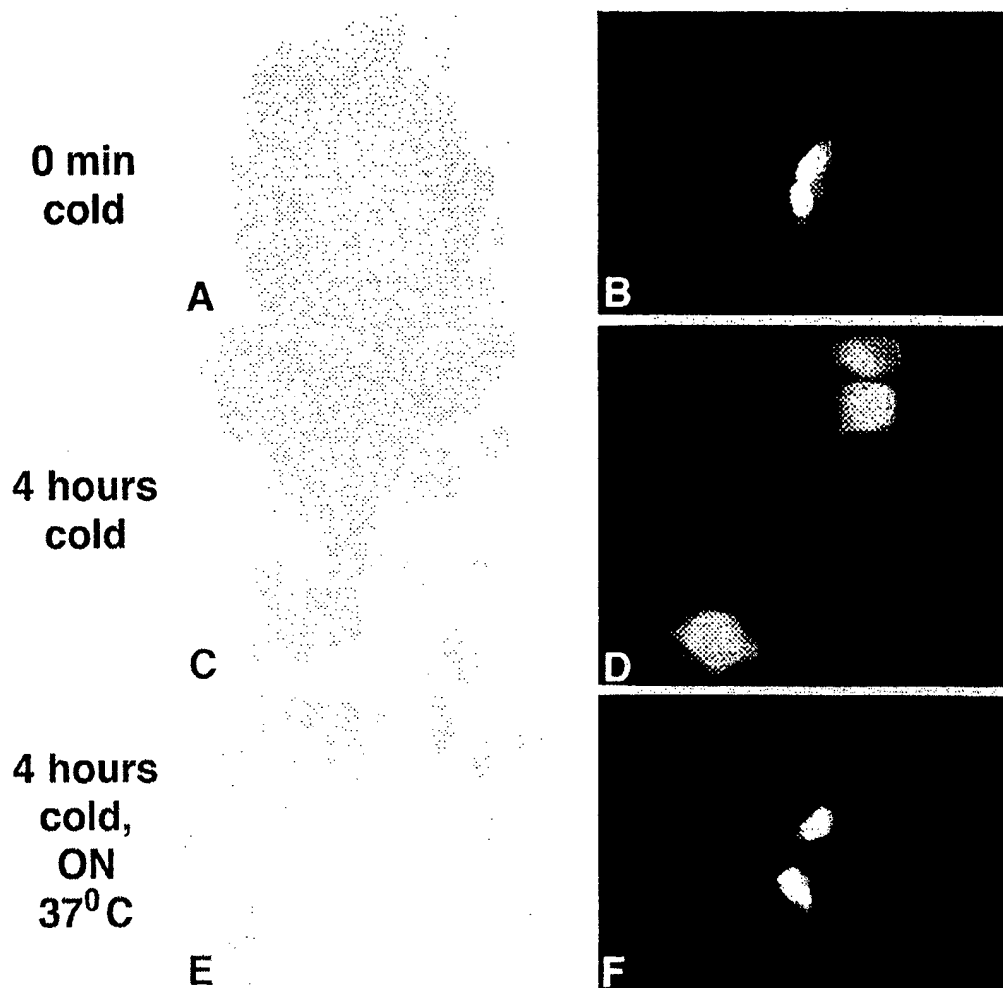
0 min
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A

45 min
ice

C
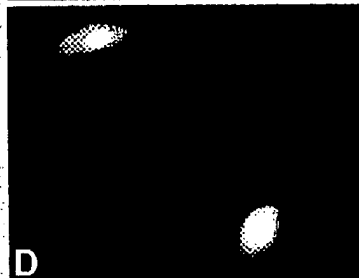
45 min
ice,
ON
37°C

E
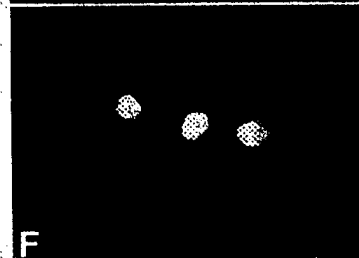
0 min
RT

G
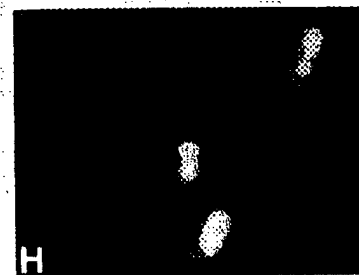
45 min
RT

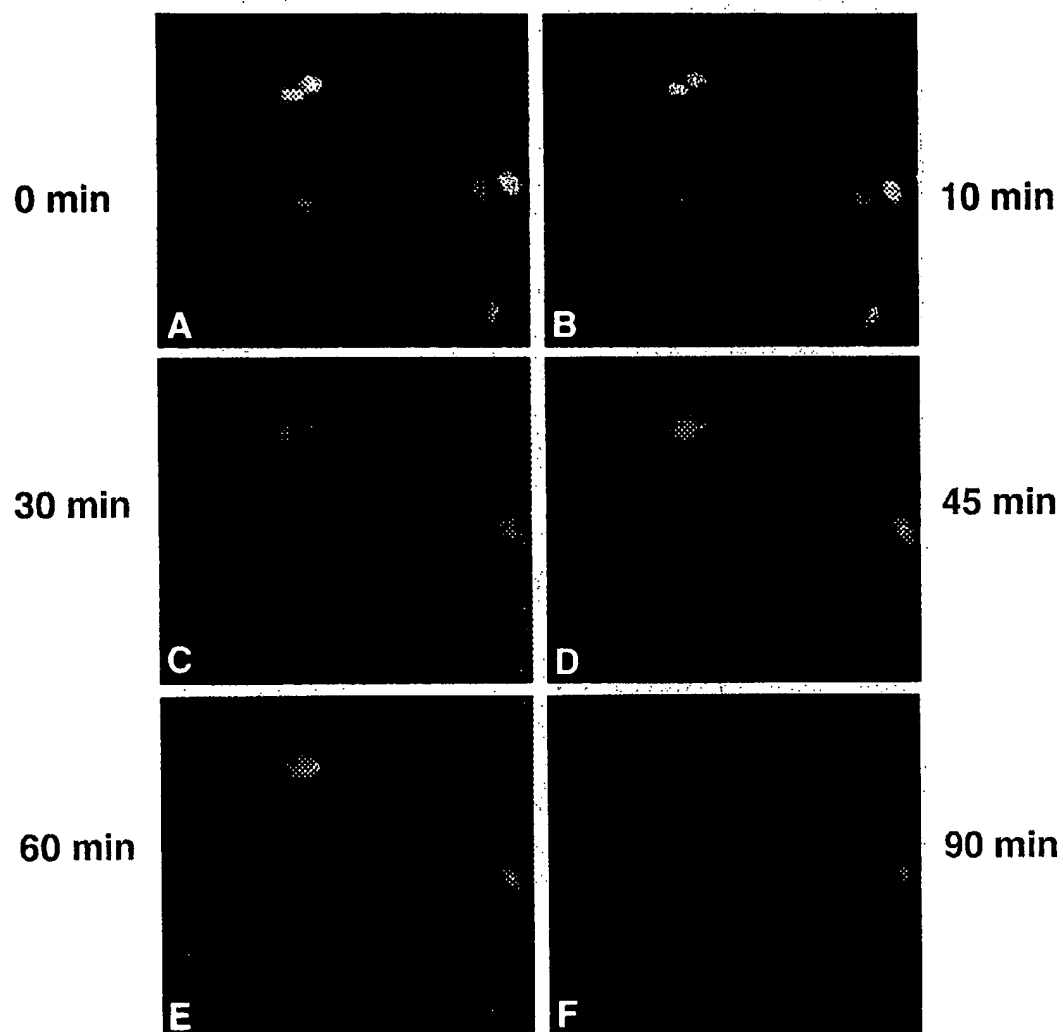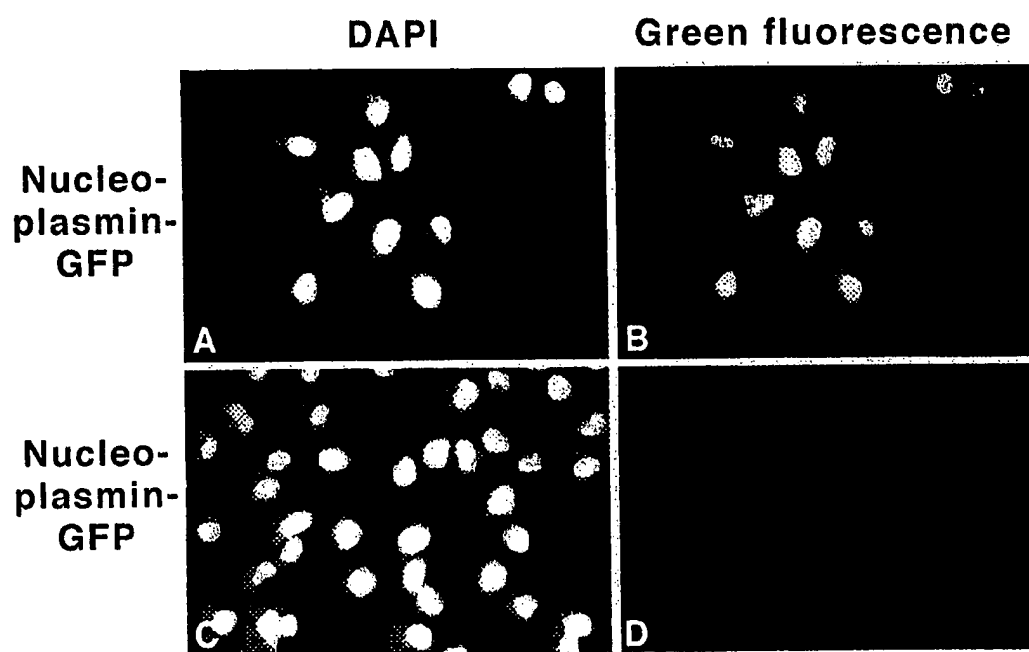I
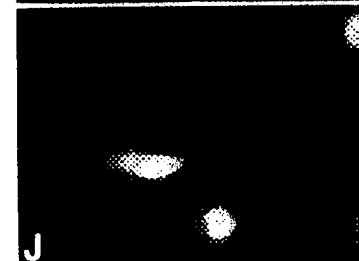
45 min
RT,
ON
37°C

K

FIG.6B

8/9

0 min

10 min

30 min

45 min

60 min

90 min

FIG_7

Fig. 8

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/62   C12N15/85   C12N5/10   G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   C07K   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHIVANI CHATTERJEE ET AL.: "Monitoring nuclear transport in HeLa cells using the green fluorescent protein" BIOTECHNIQUES., vol. 21, no. 1, July 1996, pages 62-63, XP002076910 NATICK US cited in the application see the whole document -/--	1-15

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 September 1998	22/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	PTUSHKINA M ET AL: "Intracellular targeting and mRNA interactions of the eukaryotic translation initiation factor eIF4E in the yeast Saccharomyces cerevisiae." BIOCHIMICA ET BIOPHYSICA ACTA, (1996 AUG 14) 1308 (2) 142-50. JOURNAL CODE: AOW. ISSN: 0006-3002., XP002076911 Netherlands see abstract see page 145, right-hand column, paragraph 2 - page 148, right-hand column, paragraph 2	1,2,4-15
X	WEIS K ET AL: "The conserved amino-terminal domain of hSRP1 alpha is essential for nuclear protein import." EMBO JOURNAL, (1996 APR 15) 15 (8) 1818-25. JOURNAL CODE: EMB. ISSN: 0261-4189., XP002076912 ENGLAND: United Kingdom see the whole document	1,2,6,7, 10-15
X	SCHREIBER V ET AL: "A eukaryotic expression vector for the study of nuclear localization signals." GENE, (1994 DEC 15) 150 (2) 411-2. JOURNAL CODE: FOP. ISSN: 0378-1119., XP002076913 Netherlands see the whole document	1,2,6,7, 10-15
X	WERNER BARTH ET AL.: "The yeast nucleoporin Nsp1 binds nuclear localization sequences in vitro" BIOCHEMISTRY AND CELL BIOLOGY, vol. 74, no. 3, 1996, pages 363-372, XP002076914 cited in the application see page 364, left-hand column, paragraph 5 see page 364, right-hand column, line 2 - line 3; figure 1 see page 368, right-hand column, paragraph 4	1,3,6, 10,12, 14,15

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	CHATTERJEE S ET AL: "In vivo analysis of nuclear protein traffic in mammalian cells." EXPERIMENTAL CELL RESEARCH, (1997 OCT 10) 236 (1) 346-50. JOURNAL CODE: EPB. ISSN: 0014-4827., XP002076915 United States see abstract see page 347, left-hand column, last paragraph - page 350, right-hand column, paragraph 1	1-15
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